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Thiol WR-1065 and disulphide WR-33278, two metabolites of the drug Ethyol (WR-2721), protect DNA against fast neutron-induced strand breakage

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Abstract. The main metabolites of the cytoprotective drug Ethyol (Amifostine, WR-2721) are the thiol WR-1065 and the disulphide WR-33278 (formed by the oxidation of WR-1065). Both metabolites are well-known protectors against DNA damage induced by γ-rays. Using supercoiled plasmid DNA and restriction fragments we show that they protect efficiently also in the case of fast neutrons. In anoxic conditions WR-1065 ($Z = +2$) protects by scavenging of $OH^{\bullet}$ and by 'chemical repair' (by H donation from its SH function). WR-33278 ($Z = +4$) protects by scavenging of $OH^{\bullet}$ and, in the case of the supercoiled plasmid DNA, by reducing the accessibility of radiolytic attack sites via the induction of packaging of DNA in liquid-crystalline condensates (observed by circular dichroism). Because of this second mechanism, the plasmid DNA is more efficiently protected by WR-33278 than by WR-1065, at concentration ratios > 1 drug/4 nucleotides. Moreover, using sequencing gel electrophoresis of irradiated fragments of known sequence, we show that the protection by the two metabolites is non-homogeneously distributed along the DNA sequence, with 'hot spots' of protection and with unprotected regions. Based on presented molecular modelling results we explain the sequence dependence of radioprotection by structural variations induced by the binding of the drugs.

1. Introduction

Ethyol (Amifostine) is a new promising cytoprotective drug containing the phosphorothioate WR-2721 (Capizzi and Oster 1995). In most cases, it protects specifically normal cells without diminishing the damaging effect of chemo- and radiotherapies on tumour cells (Washburn *et al.* 1974, Yuhas *et al.* 1980, Brown *et al.* 1984, van der Vijght and Peters 1994, Wasserman 1994). This interesting property is partially due to better penetration of the drug into normal than into tumour cells (Yuhas 1980). The main cellular radioprotective metabolite of WR-2721 is the thiol WR-1065 (Calabro-Jones *et al.* 1988, Smoluk *et al.* 1988). The conversion of the phosphorothioate into its thiol occurs by the dephosphorylation with alkaline phosphatase, an enzyme generally more abundant in normal tissues than in tumours (Calabro-Jones *et al.* 1985).

Strand breaks of DNA, critical radiation-induced damages, are mainly due to the abstraction of an H atom from the 4′ position of deoxyribose by the $OH^{\bullet}$ radicals produced by the radiolysis of water (Giese *et al.* 1995). The well-known radioprotective ability of thiols is explained by the scavenging of radiation induced $OH^{\bullet}$ radicals and by the 'chemical repair' of the damaged deoxyriboses of DNA, by H atom donation from the SH function (Fahey 1988, Held 1988). The most efficient radioprotectors are the positively charged thiols that concentrate close to DNA due to electrostatic attraction (Vasilescu *et al.* 1986, Zheng *et al.* 1988, Spotheim-Maurizot *et al.* 1991). It was shown that WR-1065, which has an electric charge $Z = +2$ at neutral pH (Newton *et al.* 1992), is a better radioprotector than the thiol cysteamine ($Z = +1$) in the case of irradiation of cells and of plasmid DNA with γ-rays. It is also a much better protector than the disulphide cystamine with $Z = +2$ (Aguilerra *et al.* 1992, Zheng *et al.* 1992).

Carnes and Grdina (1992) reported a significant protection by WR-2721 also against the mice neoplastic mortality induced by high LET neutrons. However, the drug seems to protect less efficiently against the cytotoxic and mutagenic effects of neutrons than against those of γ-rays (Sigdestadt *et al.* 1986, Kataoka *et al.* 1992). This can be due to a lower protection of DNA by the thiol metabolite WR-1065 against neutrons than against low LET radiations since, generally, thiols protect less efficiently from high LET than from low LET radiations (Bird 1980, Sigdestadt *et al.* 1986). In a previous work we have shown that cysteamine protects less efficiently from fast neutrons- than from γ-ray-induced DNA strand breaks only in anoxia (Spotheim-Maurizot *et al.* 1991). We explained this discrepancy by a possible competition between the 'chemical

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repair' (occurring for both radiations) and the 'fixation' of damages by the oxygen that in initially anoxic solution is produced in the secondary, dense ionization tracks of neutrons (by protons, α particles and heavy ions).

Using plasmid DNA, we evaluate in the present work the ability of the thiol WR-1065 to protect DNA from fast neutron-induced strand breaks. Anoxic conditions were employed since WR-1065 gets easily oxidized and converts into several products, from which the main one is the highly charged disulphide WR-33278 ($Z = +4$). This disulphide is also a metabolites of WR-2721 *in vivo*, but its role in the radioprotection is not yet clearly established (Smoluk *et al.* 1986, 1988, Shaw *et al.* 1994). We checked also its effect on the plasmid DNA radiolysis. The effects of WR-1065 and WR-33278 are compared with those of cysteamine and cystamine.

The radiation-induced strand breakage of DNA in solution is non-homogeneously distributed along the molecule. This modulation of radiosensitivity can be explained by sequence-dependent variations of DNA structure. For instance, the reported radioresistance of the AATT regions (Franchet-Beuzit *et al.* 1993, Isabelle *et al.* 1995, Spotheim-Maurizot *et al.* 1995a) is consistent with the reduced accessibility of the 4′ positions located in the reputed narrow minor groove of this sequence (Fratini *et al.* 1982). The radioprotection by proteins is also non-homogeneous. It occurs at and around their sites of binding to DNA. This protection can be explained by the local scavenging of $OH^{\bullet}$ radicals, by the physical screening of the binding site (inaccessibility of the radiolytic attack site) and by an eventual structural change of the DNA binding region (Franchet-Beuzit *et al.* 1993, Isabelle *et al.* 1993). The radioprotection by the positively charged thiols and disulphides can also be non-homogeneous if their binding to DNA modifies the conformation of the binding site and of the neighbouring zones. Vasilescu *et al.* (1986) have shown that the electrostatic binding of WR-1065 to DNA occurs at two neighbouring phosphates of the same strand or of opposite strands, as previously suggested for a polyamine of a closely related chemical structure, the spermine (Feuerstein *et al.* 1986). As for putrescine or spermine, the binding of WR-1065 and WR-33278 can induce distortions of the B-DNA helix (Feuerstein *et al.* 1986, Lavery *et al.* 1986) that can lead to variations of DNA radiosensitivity and radioprotection.

We have determined the distribution of the protection by WR-1065 and WR-33278 along an 80-bp restriction fragment containing two radioresistant bent AATT sequences. The results are discussed in relation to those of a molecular modelling study of DNA–WR drugs complexes.

2. Materials and methods

2.1. *Chemicals*

The pOP203 plasmid (Fuller 1982) was prepared according to Sambrook *et al.* (1989). Before irradiation, plasmid DNA was dialysed extensively against 1 mmol dm^{-3} phosphate buffer, pH 7·25. DNA was irradiated at a concentration of 50 $\mu g\,ml^{-1}$ ($1{\cdot}4 \times 10^{-4}$ mol dm^{-3} nucleotide).

The fragment of 80 bp was prepared from the plasmid DNA and labelled as previously described (Franchet-Beuzit *et al.* 1993).

Solutions of cysteamine, cystamine (Merck), WR-1065 and WR-33278 (Walter Reed Army Institute, Washington, DC, USA) were prepared in 1 mmol dm^{-3} potassium phosphate, pH 7·25 buffer. The solutions of drugs and of DNA were separately deoxygenated by flushing with pure argon, mixed and kept under argon flushing until irradiation. DNA solutions irradiated without additives were deoxygenated in the same conditions.

The possible oxidation of the thiols during the experiment was measured for cysteamine by the 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) method. In the argon-flushed solution of 0·1 mmol dm^{-3} cysteamine and at 273 K, 10% of the cysteamine became oxidized during the irradiation with 30 Gy of fast neutrons.

2.2. *Irradiations*

Irradiations were performed with fast neutrons obtained by the nuclear reaction of 34 MeV protons on a semithick beryllium target (Centre d'Etude et de Recherche par Irradiation, CNRS, Orléans). For experiments with plasmids the mean dose-rate of fast neutrons monitored with transmission chambers was 0·3 Gy/min and the dose mean lineal energy in water at the point of interest was 87·7 KeV/μm. For experiments with fragments the mean dose-rates were 10 Gy/min.

The samples were contained in polypropylene tubes (Eppendorf, 0·4 ml) immersed 1 cm below the surface of an ice bath.

2.3. *Electrophoresis*

Horizontal agarose (1·2%, Gibco BRL) minigels in Tris-acetate buffer (Tris 20 mmol dm^{-3}, sodium acetate 10 mmol dm^{-3}, EDTA 1 mmol dm^{-3}, pH 8·1) were run for 2 h at 830 Vm^{-1} at 293 K. The electrophoresis separates the supercoiled, the circular

relaxed and the linear forms of the plasmid. The fractions of the three forms in each lane were assayed, after ethidium bromide staining, using a video-camera system and the DensyLab software (Bioprobe Systems). The average number of single strand breaks (ssb) per plasmid was determined as previously described (Spotheim-Maurizot *et al.* 1991).

The sequencing gel electrophoresis of the 80-bp irradiated fragments was performed as previously described (Franchet-Beuzit *et al.* 1993). To identify the bands, Maxam–Gilbert sequencing of purines and pyrimidines of the same fragment was performed on the same gel.

Gels were fixed in a mixture of 10% acetic acid and 10% ethanol, rinsed with water, dried onto Whatman 3MM paper. They were then exposed onto PhosphorImager, which allows scanning and quantitative analysis using the ImageQuant software (Molecular Dynamics).

2.4. *Circular dichroism*

The circular dichroism spectra were recorded at 277 K with a Jobin–Yvon autodichrograph Mark V. In each case, the spectra were recorded after complete stabilization of the signal.

2.5. *Molecular modelling*

A molecular modelling study was carried out with SYBYL software (TRIPOS 6.2 (1995) St Louis, MO, USA). A DNA sequence of 30 bp was initially built up in the standard B conformation and the thiol and disulphide molecules were built separately. The thiol molecules with positive charges on its two amine groups was anchored electrostatically on the negatively charged phosphates of two successive nucleotides. Three different positions were explored, with the thiol located: (1) longitudinally in the minor groove, (2) longitudinally in the major groove and (3) transversally across the minor groove. The energy calculations were performed with the Amber force field using the Kollman's charges (Weiner *et al.* 1984). The solvent effect was implicitly taken into account by introducing a distance-dependent dielectric constant. The disulphide molecule was anchored electrostatically on the negatively charged phosphates of four successive nucleotides. The naked DNA or the DNA–WR complexes were energy minimized with the Powell method (Powell 1977). The main feature of the analysis was the minor groove width, which was measured at each pair of nucleotide as the mean distance between the H5′ atom on one strand and the H4′ atom on the other strand (Edwards *et al.* 1992).

3. Results

3.1. *Radiolysis of plasmid DNA*

Deoxygenated solutions of supercoiled plasmid DNA (4362 bp) were irradiated at 273 K with 30 Gy fast neutrons. The irradiation induced 0·02 ssb/plasmid/Gy. The number of ssb decreases when DNA is irradiated in the presence of increasing amounts of WR-1065, WR-33278, cysteamine and cystamine. The ratios of the number of ssb induced in the presence and in the absence of the drugs ($1/PF$) as a function of R (concentration of added drug/concentration of nucleotides) are shown in Figure 1. The protection efficiencies are in the order WR-1065 > cysteamine > cystamine. For WR-33278, the shape of the protection curve is different from those of the three other drugs. At low concentrations (first part of the curve) it protects less efficiently than WR-1065. At high concentrations of drug the situation is inverted.

To search for eventual WR-33278-induced conformational changes, we have performed CD measurements. Figure 2 shows the CD spectra of DNA in the presence of increasing amounts of the disulphide. We observe large changes in the spectrum from $R=$ 0·26, thus in the concentration range where radioprotection by WR-33278 overpasses that by WR-1065. The variation of the intensity of the CD signal at 260 nm and of the $1/PF$ as a function of R are shown in Figure 3.

3.2. *Radiolysis of the 80-bp restriction fragment*

Deoxygenated solutions of ^{32}P-labelled 80-bp restriction fragments were irradiated with 80 Gy fast

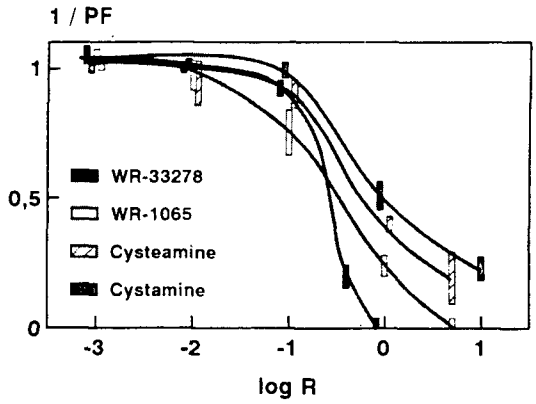


Figure 1. Variation of the inverse of the SSB protection factor (1/PF) as a function of the number of drug molecules per nucleotide, R. Irradiation, 30 Gy fast neutrons. Buffer, 1 mmol dm^{-3} potassium phosphate, pH 7·25. Curves are the best smoothing of the experimental values (averages of at least three experiments).

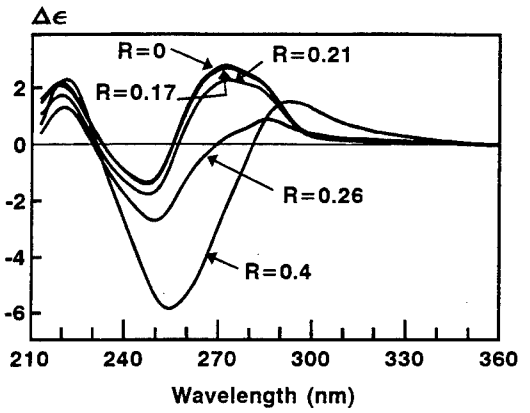


Figure 2. Modification of the circular dichroism spectra of the supercoiled plasmid DNA in the presence of increasing amounts of WR-33278. DNA was in 1 mmol dm^{-3} potassium phosphate buffer, pH 7·25.

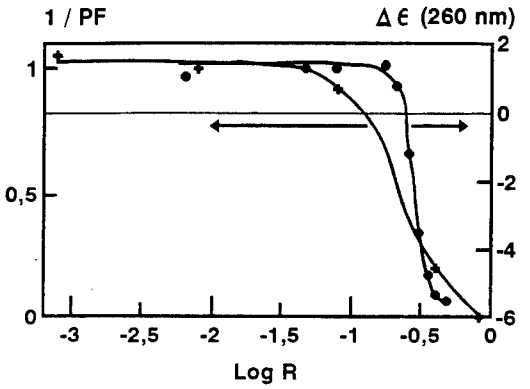


Figure 3. Variation of the circular dichroism signal at 260 nm (circles) and the SSB protection curves in 1 mM potassium phosphate buffer (crosses) as a function of the WR-33278 concentration.

neutrons. The sequencing gel electrophoresis of the irradiated fragments shows the induction of frank strand breaks at all nucleotide sites. The probability of breakage varies along the fragment. The breakage pattern of DNA (probability of breakage at each nucleotide site) reveals the previously reported relative radioresistance of the AATT sequences. When the fragments were irradiated in the presence of drugs, the breakage patterns are modified. Figure 4 shows as an example the breakage patterns of DNA irradiated without additive and in the presence of 0·1 WR-1065/nucleotide.

The protection patterns (ratio of the breakage probabilities for DNA with and without drug) calculated from the breakage patterns of 'naked' DNA and of DNA irradiated in presence of the drugs are presented in Figures 5 and 6. They reveal a non-homogeneous protection by both WR drugs.

For WR-1065 at $R=0{\cdot}1$, two 'hot spots' of protection (M and O) are observed along the analysed sequence, the strongest being at the sequence M. A protection lower than the average value is observed for the sequence N. At $R=1$ the pattern is modified and a new, but less intense, 'hot spot' of protection appears at a TGT sequence (P) and the low-protected region is shortened and shifted in the 5′ direction to the sequence Q (Figure 5).

The pattern for WR-33278 at $R=0{\cdot}1$ shows a globally low protection. Protection occurs at the sequence X and at a GTC triplet (Y). The rest of the analysed sequence is not protected. At $R=1$, almost the entire fragment is protected. The strongest protection occurs at the TCCT sequence belonging to the region X. One nucleotide every 9–12 nucleotides is unprotected. This renders a wave-like shape to the protection pattern (Figure 6). The period does not change with increasing disulfide concentration.

3.3. *Molecular modelling*

The molecular modelling of a 30-bp sequence of our 80-bp fragment shows that the two AATT zones exhibit a minor groove restricted width. This result is in good agreement with the crystallographic data (Fratini *et al.* 1982). The zones with a narrow minor groove are the zones of low probability of radiation induced strand breakage in the naked DNA (Figure 7).

We have constructed DNA–WR-1065 complexes and observed that the longitudinal location of the thiol in the minor groove (around $-209\,kJ\cdot mol^{-1}$) is energetically more favourable than that in the major groove (around $-167\,kJ\cdot mol^{-1}$) and than the transversal location across the minor groove (around $-155\,kJ\cdot mol^{-1}$).

The DNA–WR-1065 complex with the thiol molecule electrostatically anchored along the minor groove at T24C25 site (belonging to the 'hot spot' of protection M; Figure 5, top) shows a narrowing of the minor groove in and around the binding zone. Moreover the thiol masks the H4′ atoms of the nucleotides to which it is anchored (Figure 8). On the contrary, the minor groove is widened when one molecule of WR 1065 is anchored at the T7G8 site (belonging to the only slightly protected region N; Figure 5, top). Since in this case the thiol is deeply inserted in the groove, the H4′ atoms of the nucleotide to which it is anchored, are not masked by the ligand (Figure 9). When six molecules of thiol are bound to the 30 bp oligonucleotide, the conformation of the complex is different: the minor groove in the zone around T7G8 (which at high concentration belongs to the 'hot spot' of protection P; Figure 5) becomes also narrower than in the naked DNA (data

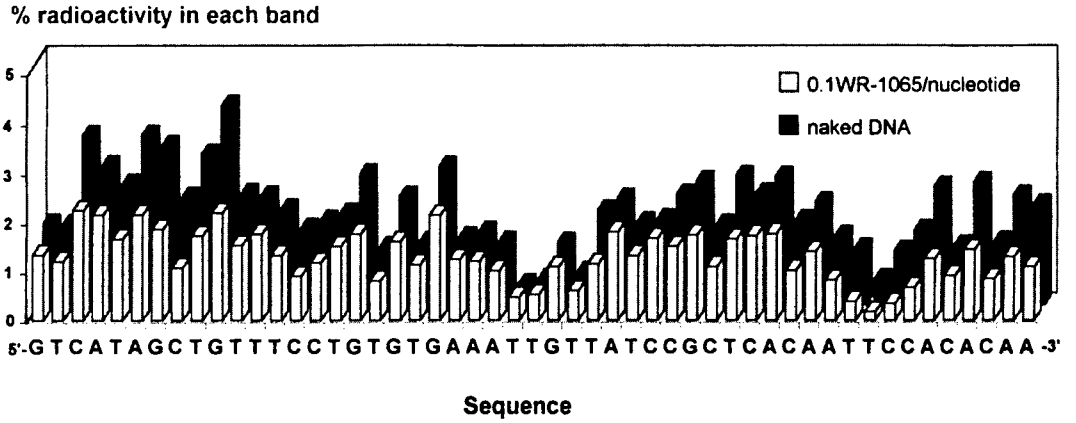
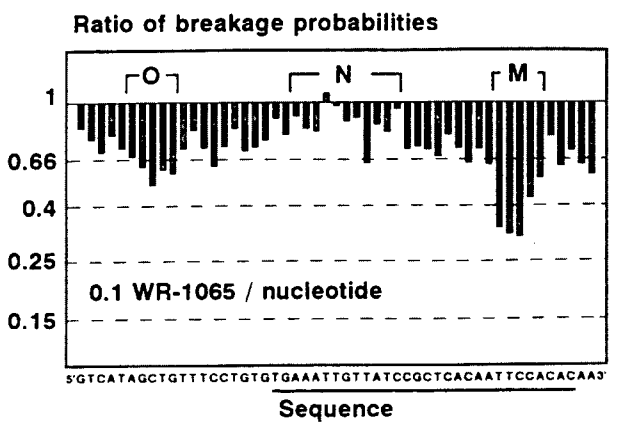
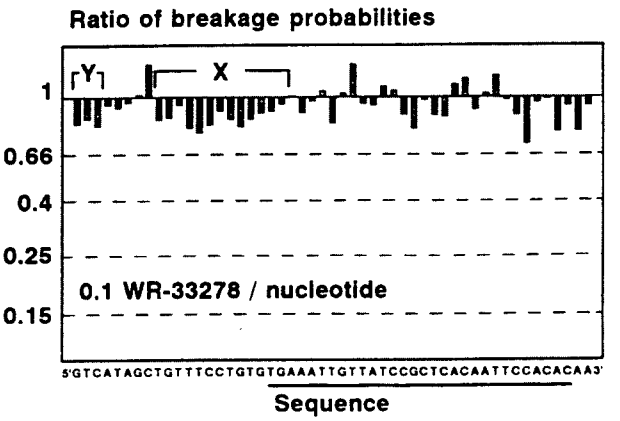
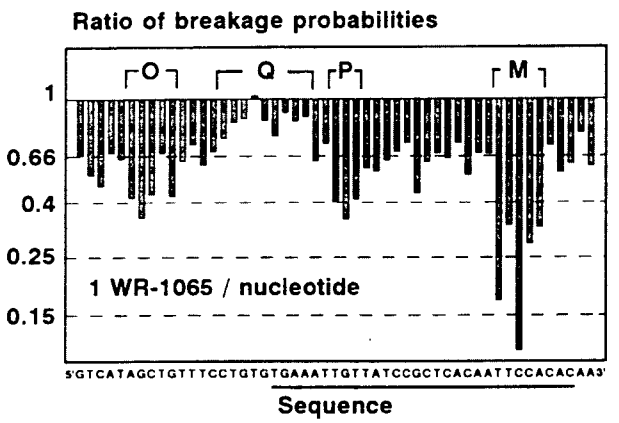


Figure 4. Probability of strand breakage at each nucleotide irradiated in the presence (clear bars) and in the absence (dark bars) of 0·1 WR-1065/nucleotide along a part of the 80 bp DNA fragment. Irradiation dose, 80 Gy fast neutrons. Anoxic conditions. The results are those of one typical experiment out of five rendering similar patterns. The experimental error in the determination of area of peaks in the densitogram of gels is < 3%.







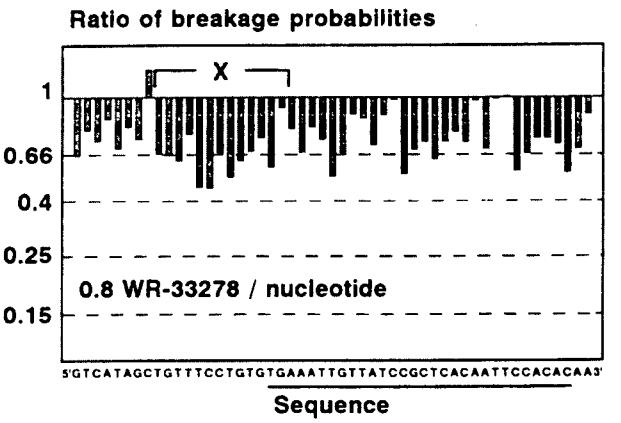


Figure 5. Ratios of the probability of strand breakage at each nucleotide along a part of the 80 bp DNA fragment irradiated in the presence and in the absence of WR-1065. Irradiation dose, 80 Gy of fast neutrons. Anoxic conditions. The results are those of a typical experiment out of five rendering similar patterns. The experimental error in the determination of the areas of peaks in the densitogram of gels is < 3%. Underlined, the sequence of the modelized 30 bp fragment.

Figure 6. Ratios of the probability of strand breakage at each nucleotide along a part of the 80 bp DNA fragment irradiated in the presence and in the absence of WR-33278. Irradiation dose, 80 Gy fast neutrons. Anoxic conditions. The results are those of a typical experiment out of five rendering similar patterns. The experimental error in the determination of the areas of peaks in the densitogram of gels is < 3%. Underlined, the sequence of the modelized 30 bp fragment.

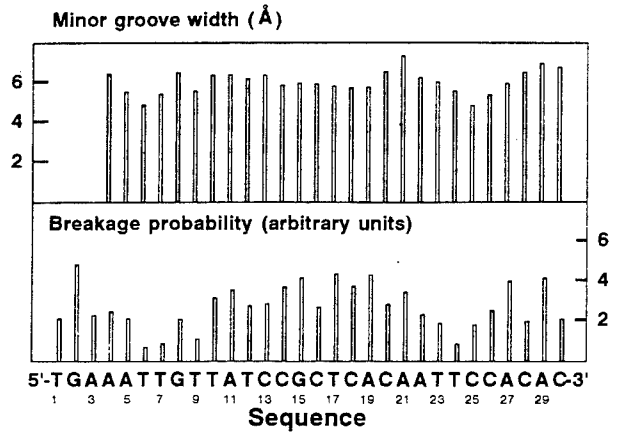


Figure 7. Variation of the minor groove width of a naked 30 bp DNA calculated by molecular modelling (up) and the probability of strand breakage at each nucleotide along the irradiated naked DNA, from Figure 4 (down).

not shown). In all discussed locations, the SH function, which moreover can rotate around the terminal C-S, is close enough to the 4′ position to allow the H donation and thus the chemical repair.

We have modelled the DNA–WR-33278 complex with one disulphide molecule electrostatically anchored along the minor groove at T24C25C26A27 site. This site belongs in this case to an unprotected region (in the case of WR-1065 it belongs to the 'hot spot' of protection M). The final structure exhibit a widening of the minor groove in and around the binding zone. The disulphide molecule is deeply inserted in the groove and the H4′ atoms of the nucleotide to which it is anchored are not masked.

When six molecules of disulphide are bound to the 30 bp oligonucleotide, the minor groove width is strongly reduced all along the 30 bp fragment with minima at the sites of anchorage and maxima at the uncovered nucleotides (periodic variation with period of 4 bp). The total length of the fragment is decreased by 0·18 nm (0·006 nm/bp). When eight molecules are bound, the variation of the minor groove width exhibit a periodicity of 3 bp.

4. Discussion

4.1. *Mechanisms of protection*

The anoxic radioprotection by the metabolites WR-1065 and WR-33278 of the drug WR-2721 can be explained by:

(i) the scavenging of $OH^{\bullet}$ radicals,
(ii) the 'chemical repair' of damaged sugars (for the thiol WR-1065),
(iii) the packaging of plasmid DNA into a radioresistant condensate (for high concentrations of the disulfide WR-33278).

4.1.1. *Scavenging of $OH^{\bullet}$ radicals and 'chemical repair' of damaged sugars* The rate constant of the reaction of WR-1065 with $OH^{\bullet}$ radicals ($9{\cdot}2 \times 10^9$ dm^3 mol s^{-1}; Ward and Mora-Arellano 1984) is lower than that of cysteamine ($1{\cdot}8 \times 10^{10}$ dm^3 mol s^{-1}; Buxton *et al.* 1988). Nevertheless, WR-1065 protects more efficiently than cysteamine: at 1 drug/nucleotide, $PF_{\text{WR-1065}} \approx 2 \times PF_{\text{cysteamine}}$ (Figure 1). This can be due to its higher charge and thus to its higher degree of condensation around DNA (Manning 1978). Thus WR-1065 can more efficiently repair the damaged sugars and scavenge the $OH^{\bullet}$ radicals reaching DNA. Cystamine is a disulphide with the same positive charge as WR-1065 ($\mathcal{Z} = +2$), with a similar chemical structure (diamine), but missing the SH function. Although its reaction rate with $OH^{\bullet}$ radicals is the same as that of cysteamine (Buxton *et al.* 1988) and its charge is higher, it protects slightly less than cysteamine since it is unable to 'chemically repair' the damaged sugars. Thus, in spite of its lower $OH^{\bullet}$ scavenging ability, WR-1065 is the best radioprotector. The mechanism of protection by scavenging of radicals and by 'chemical repair' was already proposed by Zheng *et al.* (1988) for the protection by WR-1065, cysteamine and cystamine against γ-rays in aerobic conditions.

The results of molecular modelling are in good agreement with the mechanisms of protection discussed above. The energetically most favourable location of the thiol and of the disulphide in the minor groove allows the interference with the initial damaging process, the abstraction of a H atom from the 4′ position situated in the minor groove (by $OH^{\bullet}$ radicals) with formation of a 4′ sugar radical. Therefore they can efficiently scavenge the $OH^{\bullet}$ radicals and repair the sugar radical. In addition, the drug molecules can protect particular sequences also by masking the 4′ positions and by narrowing the minor groove. Such sequence-dependent effects can account for the variations of protection along the molecule (see discussion below).

4.1.2. *Packaging of DNA into a radioresistant condensate.* Although cystamine protects plasmid DNA less efficiently than cysteamine, WR-33278 protects more efficiently than WR-1065 at $R > 0{\cdot}25$. To explain the radioprotection by WR-33278 we propose, together with the scavenging of $OH^{\bullet}$ radicals, another type of mechanism which involves also the charge of the drug, $\mathcal{Z} = +4$. The modifications of the shape and intensity of CD spectra at drug concentrations that strongly radioprotect are similar to those previously

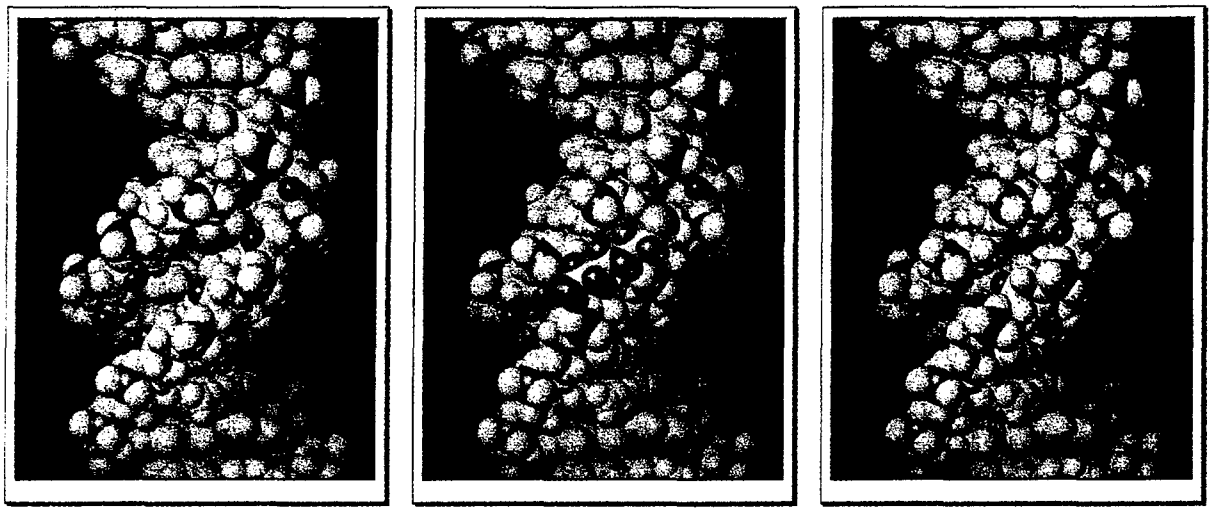


Figure 8. A part of the model of the 30 bp DNA with one WR-1065 molecule bound at the T24C26 sequence. (left) Naked DNA, (centre) DNA-WR 1065 complex, (right) DNA-WR 1065 complex with hidden thiol. Colours: P, orange; H'4, magenta; S, yellow; N, blue; H atoms of the thiol, cyan; all other atoms, white.

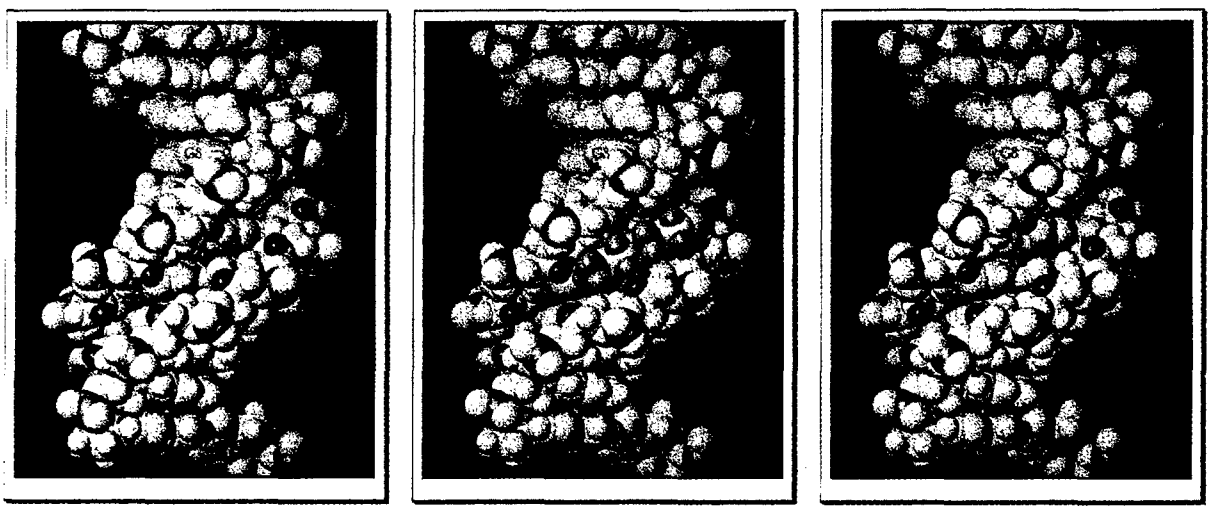


Figure 9. A part of the model of the 30 bp DNA with one WR-1065 molecule bound at the T7G8 sequence. (left) Naked DNA, (centre) DNA-WR 1065 complex, (right) DNA-WR 1065 complex with hidden thiol. Colours: P, orange; H'4, magenta; S, yellow; N, blue; H atoms of the thiol, cyan; all other atoms, white.

observed for other radioprotective molecules with closely related chemical structures, the polyamines spermidine ($\mathcal{Z}=+3$) and spermine ($\mathcal{Z}=+4$) (Spotheim-Maurizot *et al.* 1995b, Newton *et al.* 1996). The modifications of CD spectra (at drug concentrations corresponding to an extensive neutralization of the negative charge of DNA) may reflect the same important conformational changes as for the polyamines: the formation of liquid–crystalline structures of toroidal or rod-like shape. The packaging of DNA molecules longer than 400 bp into such condensed structures is triggered by the extensive neutralization of the negative charges of DNA (92% for the ligands with $\mathcal{Z}=+3$ and 94% for ligands with $\mathcal{Z}=+4$; Manning 1978) that render possible intra- and intermolecular interactions (Bloomfield 1991, and references herein). We have explained the 'simultaneous' radioprotection of DNA by spermidine or spermine, by the reduction of the accessibility of $OH^{\bullet}$ radicals to the attack sites (nucleotides) in the condensates. We suggest here that WR-33278 radioprotects DNA by the same mechanism. Shigematsu *et al.* (1994) have shown similarities in the radioprotection by spermine and WR-33278 against mutagenesis at the *hprt* locus in Chinese hamster AA8 cells.

In the case of fragments, WR-33278 protects less efficiently than WR-1065 even at high concentration. This can be due to the fact that the 80 bp

oligonucleotides is too short to condense into the same type of highly radioresistant structure as the plasmid (Bloomfield 1991).

4.2. *Sequence-modulated protection*

The electrostatic binding of a small positively charged molecule to one or more neighbouring phosphates (on the same strand or on opposite strands) is *a priori* non-specific and therefore it is expected to be sequence-independent. Nevertheless, it was shown that the polyamines bind preferentially to particular sequences. The preferential binding was explained by sequence-dependent variations of DNA structure and electrostatic potential (Feuerstein *et al.* 1986, Lavery *et al.* 1986).

The non-homogeneous protection by WR-1065 and WR-33278 cannot be simply explained by a sequence-dependent binding to DNA, since no clear sequence similarity appears between the observed 'hot spots' of protection.

Our results of molecular modelling suggest another (or an additional) explanation of the sequence-dependence of the radioprotection. It would be due to structural variations induced by the binding of the drugs and to the eventual masking of radiolytic attack sites. The electrostatic binding of WR-1065 to two nucleotides belonging to the 'hot spot' M, in the energetically most favourable location (longitudinally in the minor groove), triggers the narrowing of the minor groove at and around the bound nucleotides, and masks the H4′ position of the bound nucleotides (Figure 8). Both effects can explain the increased radioprotection in this zone.

The low protection of the region N can be explained by the increase of accessibility of the 4′ positions in the minor groove widened by the deep insertion of the thiol. Moreover these positions are not masked by the thiol as in the 'hot spot'. The deep insertion can also diminish the radioprotective effect of $OH^{\bullet}$ scavenging (Figure 9).

The modification of the radioprotection pattern with increasing thiol concentration can be explained by a concentration-dependent conformational change of the helix (Vasilescu *et al.* 1986, Mallet *et al.* 1994). Moreover, Lavery *et al.* (1986) have shown modifications of the binding-induced distortion with increasing the concentration of putrescine. Such effects can either increase or decrease the radiosensitivity of the affected region.

According to our results, at high thiol concentration, the protection of the region P (unprotected at low concentrations) can be explained by the narrowing of the minor groove observed in the model of the 30 bp DNA with six bound thiols (see § 3). The emergence of a new hot spot of protection, at a TGT triplet may be related to the flexibility of this sequence (Folta-Stogniew and Russu 1995) and thus to its facility to undergo structural changes.

The lack of protection of certain zones by the deep insertion of the disulphide WR-33278 can be explained, like in the case of the WR-1065, by the widening of the minor groove, by the lack of masking the H4′ atoms and by the reduced effect of $OH^{\bullet}$ scavenging (see § 3).

The protection by high concentrations of WR-33278 involves the calculated narrowing of the minor groove all along the model of DNA with six disulphide molecules (see § 3).

The molecular modelling does not account for the constant periodicity of 10 bp of the wave-like shape of the protection pattern. This periodicity recalls the one observed in the case of the 146 bp DNA of irradiated core nucleosome (Franchet *et al.* 1993) or of shorter DNA fragments irradiated with bound charged proteins (Isabelle *et al.* 1993) or with compounds such as distamycine (experiments with chemically produced $OH^{\bullet}$ radicals; Churchill *et al.* 1990) and polyamines (spermidine and spermine; Savoye *et al.* in preparation). The common point of all these systems is the extensive symmetrical or asymmetrical neutralization (until 94% in the case of spermine and WR-33278) of the negative charge of the phosphates. According to the hypothesis of Manning (1989) such uncharged DNA, if >60 bp, would contract along its axis (0·1 Å/bp), buckle and form a bent 'spring-like' structure. Our results of modelling agree with the contraction, since a shortening of the helix is observed with WR-33278 (0·06 Å/bp). It is possible that, in the proposed regularly bent structure, the width of the minor groove varies with a period of 10 bp corresponding to the pitch of B-DNA. This will lead to a periodically variable accessibility of the H4′ atom and thus will explain the observed wave-like pattern of radioprotection by WR-33278 and, may be, by other charged molecules.

5. Conclusion

The two studied metabolites of Ethyol (Amifostine, WR-2721), the thiol WR-1065 and the disulphide WR-33278 formed by the oxidation of WR-1065, protect efficiently against the DNA strand breakage induced by fast neutrons. The fact that at relatively high concentrations WR-33278 is an even better protector than WR-1065 can contribute to the explanation of the differential radioprotection of normal and tumour cells by Ethyol (WR-2721). Since normal tissues are better vascularized (and thus better oxygenated), more WR-33278 may be generated in

the normal than in tumoral cells. Thus normal cells can be better protected than the tumour cells.

Moreover, the protection by the two metabolites of Ethyol is non-homogeneously distributed along DNA: some sequences are more efficiently protected than others. We explain this sequence-dependence by various structural changes induced by the binding of the two main metabolites.

The thiol-induced and the even stronger disulphide-induced modification of DNA structure can also influence (diminish) the subsequent binding of some anti-tumour drugs. This may explain why Ethyol is not only a good radioprotector but also an efficient cytoprotector in the chemotherapy with a large variety of drugs (Capizzi and Oster 1995).

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